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# Lipase-catalyzed reactions in organic and supercritical solvents: application to fat-soluble vitamin determination in milk powder and infant formula

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**Abstract**

The aim of this study was to thoroughly investigate the possibility of using enzyme catalyzed hydrolysis and alcoholysis of ester bonds in vitamin A and E esters to facilitate their determination in different food formulas. Two vitamin esters, retinyl palmitate and α-tocopheryl acetate were used as model compounds and two food formulas, milk powder and infant formula, were used as model matrices. Six lipase preparations and one esterase preparation were investigated in the solvents di-isopropyl ether, hexane/ethanol and supercritical carbon dioxide containing ethanol. Three of the enzyme preparations, lipases from *Candida antarctica* (Novozyme 435), *Rhizomucor miehei* (Lipozyme IM) and *Pseudomonas cepacia*, showed considerably higher activity toward retinyl palmitate than the other four enzyme preparations. There was no observed activity with α-tocopheryl acetate using any of the enzyme preparations. Novozyme 435 showed highest activity in supercritical fluid and generally larger tolerance to variations of the investigated parameters. Using this enzyme preparation in supercritical carbon dioxide containing 3 vol% ethanol and 0.03 vol% water at 366 bar and 80°C, quantitative conversion of retinyl palmitate to retinol was obtained. These conditions were then used for simultaneous lipase-catalyzed reaction and extraction of vitamin A and E from milk powder and infant formula. The developed supercritical fluid extraction method using immobilized *Candida antarctica* preparation seems to be more beneficial to the oxidation prone vitamins A and E compared to extraction methodologies based on alkaline saponification, resulting in comparatively higher recoveries. 

*Keywords:* Alcoholysis; Hydrolysis; Lipase; Organic solvent; Retinyl palmitate; SFE

## 1. Introduction

Determination of fat-soluble vitamins in food samples commonly includes hot saponification followed by liquid-liquid extraction [1,2]. However, these conventional methodologies are laborious and solvent consuming, and the alkaline environment during the saponification step promotes oxidative degradation of the labile vitamins. Supercritical fluid extraction (SFE) using carbon dioxide as extraction solvent is a faster and environmentally more beneficial technique. We have previously shown that vitamins A and E can accurately be determined in milk powder, milk, liver paste and minced meat using SFE followed by saponification and final analysis by RP-HPLC with UV and fluorescence detection [3,4]. Others have used SFE for determination of vitamin $K_1$ in infant formula [5] and for retinyl palmitate in calf liver [6] and cereal products [7].

The advantages of using a saponification step, which distinguishes our methodology [3,4] from others [5–7] employing SFE for fat-soluble vitamin determination, are higher selectivity and facilitated chromatographic determination. The possible degradation of vitamins during the alkaline saponification in our approach could be reduced to a low level for vitamins A and E by adding an antioxidant to the reaction mixture [8].

One possible way of avoiding harsh alkaline saponification procedures is to use enzymes for cleavage of ester links in vitamins and in coextracted lipids to improve the chro-

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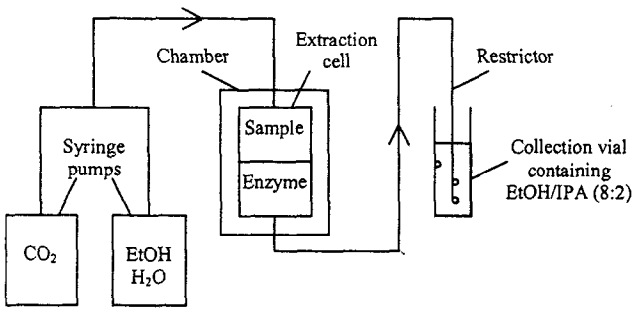


Fig. 1. Flow schematic of the SFE instrument.

matographic performance. Lipases have previously been used to isolate fat-soluble vitamins in aqueous buffer solutions followed by conventional solvent extraction. Vitamins A, D, E and $K_1$ have been isolated from milk powder, infant formula and other dairy products [9,10], as well as vitamin $K_1$ solely from milk and infant formula [11].

In a recent paper [12] we demonstrated for the first time in an analytical context that immobilized *Candida antarctica* lipase could be used in the high pressure extractor of an SFE system for hydrolysis of vitamin A esters as well as coextracted lipids before determination of vitamins A and E in different food products. The developed method utilized simultaneous extraction and reaction in a fully automated SFE system with supercritical carbon dioxide containing ethanol and water.

In this paper, using the same SFE system, we have investigated in more detail the possibility for enzymatic hydrolysis of retinyl palmitate and α-tocopheryl acetate using six different lipase preparations, representing the major groups of lipases [13], as well as a pig liver esterase preparation. Effects of different water concentrations and temperatures on the enzymatic reaction were investigated, and the optimized conditions found were used for the determination of vitamins A and E in milk powder and infant formula.

## 2. Materials and methods

### 2.1. Materials

*Supercritical fluid extractor.* A fully automated instrument of model Isco SFX 3560, Isco Inc. (Lincoln, USA) was used for the SFE experiments. It consisted of two pumps (model 260D and 100DX) that delivered carbon dioxide and modifier via a T-connection to the heated extraction chamber, as schematically illustrated in Fig. 1. In this work the modifier used was a mixture of dry ethanol and specific amounts of water. Pressure and flow rate of the fluid was automatically maintained by a restrictor, which was positioned in a collection vial containing solvent during the extraction. The instruments carousel could be loaded with 24 extraction cells and 24 collection vials, of 10 and 20 ml volume, respectively.

*HPLC system.* A Varian Star HPLC System consisting of a gradient pump 9010 equipped with a Dynamax mixer, an autosampler 9100 and a UV-VIS detector 9050, Varian AB (Solna, Sweden) was used. A ThermoSeparation FL2000 fluorescence detector, ThermoQuest (San Jose, CA), was connected on-line with the UV-VIS detector. The chromatographic column was a reverse phase column (Merck LiChrospher® RP-18, 5 μm, 250*4 mm). A Borwin chromatographic software from JMBS Developpements (Grenoble, France) was used for peak integration.

*Spectrophotometer.* An LKB 4050 UV/Visible (Stockholm, Sweden) was used for purity checking of vitamin standard solutions.

*Solvents.* HPLC grade methanol, isopropyl alcohol and hexane from Merck (Darmstadt, Germany), ethanol (99.7%) from Primalco Oy (Rajamäki, Finland), di-isopropyl ether (DIPE) from Acros Organics (Geel, Belgium) and Milli-Q water were used in the procedures.

*Chemicals.* Hydromatrix wet support matrix (SFE Grade) was obtained from Sorbent (Frölunda, Sweden), 2,6-di-tert-butyl-4-methylphenol (BHT, >99%) from Aldrich, Sigma-Aldrich Sweden AB (Stockholm, Sweden), potassium carbonate ($K_2CO_3$) from Merck (Darmstadt, Germany), potassium sulphate ($K_2SO_4$) from Fisher Scientific (Loughborough, UK) and lithium chloride (LiCl) from Acros Organics (Geel, Belgium). Polypropylene powder, Accurel EP-100, was a gift from Akzo (Obernburg, Germany). All chemicals used were of analytical grade.

*Gases.* Nitrogen (≥99.9995%), carbon dioxide (≥99.998%) and carbon dioxide used for cryogenic cooling were obtained from AGA Gas (Sundbyberg, Sweden).

*Enzymes.* Novozyme 435 (immobilized *Candida antarctica* lipase type B, CALB, 10.000 Propyl Laurate Units/g) and Lipozyme RM IM (immobilized *Rhizomucor miehei* lipase, RML, 6.1 BAUN/g) were generous gifts from Novo Nordisk A/S (Bagsvaerd, Denmark). Crude lipases from *Rhizopus oryzae* (ROL) and *Pseudomonas cepacia* (PCL) were kind gifts from Gist-Brocades S.A. (Delft, The Netherlands) and Amano Enzyme Europe Ltd. (Milton Keynes, UK), respectively. Porcine pancreatic lipase (PPL, 100.000–400.000 units/g in hydrolysis of olive oil), *Candida rugosa* lipase (CRL, 100.000–400.000 units/g in hydrolysis of olive oil) and Pig liver esterase (PLE, 15.000 units/g in hydrolysis of ethyl butyrate) were crude enzymes obtained from Sigma, Sigma-Aldrich Sweden AB (Stockholm, Sweden).

*Vitamin standard solutions.* All-trans-retinol, α-tocopherol, α-tocopheryl acetate, retinyl palmitate and vitamin $D_2$ were obtained from Fluka Chemie AG (Buchs Switzerland) and γ-tocopherol and δ-tocopherol were purchased from Merck (Darmstadt, Germany). Standard solutions were prepared by diluting with ethanol, and their concentrations were determined spectrophotometrically [14–16].

*Food samples.* Milk powder and infant formula obtained from local producers were chosen as samples. Samples from the same batch had previously been used in an Intercomparison study concerning fat-soluble vitamin determination [17]. The recoveries presented in this work were calculated by comparison with average values obtained in the Intercomparison study, where ten laboratories performed extractions using SFE followed by alkaline saponification and two laboratories used conventional extraction techniques that also included saponification. After removing outliers, the calculated average values were considered as reference values (see last row of Table 4).

### 2.2. Methods

*Chromatographic conditions.* Gradient elution was applied in order to separate the considered vitamins within reasonable analysis time. The mobile phase consisted of A: methanol and B: isopropyl alcohol, mixed as follows: 0–25 min. A (100%), 25–35 min. A (100%)—A/B (80:20), 35–48 min. A/B (80:20) and finally 48–60 min. reequilibration of the system with A (100%). Detection was performed using a variable UV detector programmed as follows: 0–8 min. 325 nm (retinol), 8–14 min. 265 nm (vitamin $D_2$), 14–17 min. 292 nm ($\alpha$-tocopherol), 17–25 min. 284 nm ($\alpha$-tocopheryl acetate) and 25–48 min. 325 nm (retinyl palmitate). A fluorescence detector was connected on-line with the UV detector for determination of $\alpha$-, $\gamma$- and $\delta$-tocopherol. The wavelengths were set to 226 and 330 nm for excitation and emission, respectively.

*Immobilization of enzymes.* The crude enzyme preparations were immobilized on to a polymeric support material, Accurel EP-100, following a procedure described by Gitlesen et al. [18]. The amount of adsorbed protein was calculated by determining the concentration of solubilized protein, using the Bradford method with BSA as standard protein.

*Experiments in organic solvents.* Experiments were performed using two different organic solvents: water-saturated di-isopropyl ether (DIPE), which contains approximately 1.2% (w:w) of water at 20°C, and hexane/ethanol (87.5/12.5, v:v). The water activity of the hexane/ethanol mixture, also containing retinyl palmitate (ca 50 $\mu$g, hence 10 nmol) and $\alpha$-tocopheryl acetate (ca 200 $\mu$g, hence 400 nmol), was set to 0.11 over night at room temperature in closed vessels using LiCl [19]. The reason for using a higher concentration of $\alpha$-tocopheryl acetate than retinyl palmitate is that the former gives approximately 50 times weaker UV absorption than vitamin A and retinyl palmitate. The immobilized enzymes were similarly equilibrated over night to water activities of 0.97 and 0.11 using $K_2SO_4$ and LiCl, respectively [19]. When DIPE was used as solvent, the vitamin esters were added just before starting the reaction, due to their limited stability in solvents containing water [20]. The reactions were started by adding 20 mg of enzyme preparation to each 4-ml portion of organic solvent. The reactions were performed in closed amber vials at room temperature and with continuous shaking. Aliquots of 0.5 ml were taken after 2, 6 and 24 hours of reaction for analysis of formed vitamin alcohols. Recoveries of retinol (vitamin A alcohol) were calculated using vitamin standard solutions. The starting concentrations of the unreacted vitamin esters at the different time intervals were determined using blank mixtures (n = 3), where the reaction conditions were identical except that enzymes were excluded. The reason for this operation is that the vitamin esters might be partly degraded during the course of the reaction, which would give erroneous values of the recoveries if compensations were not made. Vitamin $D_2$ was included as internal standard to correct for possible volume decrease due to leakage of solvent vapour during the reaction.

*Experiments in supercritical carbon dioxide.* 0.5 g of immobilized enzyme was mixed with 1.0 g of hydromatrix and transferred to the extraction cell, where it formed a 3 cm long plug of porous structure. This enzyme mixture was cleaned with supercritical carbon dioxide to remove liposoluble components from the enzyme preparation (see also Results). The supercritical conditions used for cleaning were: 5 min. static extraction (pressurized chamber and almost zero flow rate) followed by 30 min. dynamic (pressurized chamber and a constant flow rate), at a flow rate of 2.0 ml/min. Supercritical carbon dioxide of pressure 260 bar and temperature 60°C (density 0.8 g/ml), containing 3 vol% of ethanol and 0.15 vol% of water, was used. The water was always added to dry ethanol, i.e. the ethanol contained 5 vol% of water in order to provide the supercritical carbon dioxide with 0.15 vol% of water. The water activity ($a_w$) of the enzyme preparation was set over night at room temperature to 0.43 in a desiccator containing water saturated with $K_2CO_3$ [19]. The enzymatic extraction/reaction was performed by filling up the extraction cell with hydromatrix ($a_w$ = 0.43), and then adding 1 ml of 99% ethanol containing retinyl palmitate (ca 10 $\mu$g, hence 2 nmol) and $\alpha$-tocopheryl acetate (ca 50 $\mu$g, hence 100 nmol). The direction of the flow was from the sample towards the enzyme bed. The supercritical conditions used for extraction/reaction were basically chosen according to previous experiences [12]: 5 min. static extraction and 15, 30, 30 and 45 min. steps of dynamic extraction. The collection was achieved in four separate vials containing 10 ml of ethanol/isopropyl alcohol (8/2, v/v), with BHT added as antioxidant and vitamin $D_2$ as internal standard for volume corrections. The supercritical solvent used was carbon dioxide containing 3 vol% of ethanol and 0.03–0.15 vol% of water, with the extraction performed at 40–80°C and a density of 0.8 g/ml. The flow rate was 0.5 ml/min during the first 75 minutes, where the overall kinetic was mainly limited by the enzymatic reaction, and 1.0 ml/min during the last 45 minutes, where the substrate concentration was much lower and supercritical extraction kinetic was the limiting factor. Moreover, the modifier flow of ethanol and water was set to 0 the last 10 minutes of the extraction in order to avoid possible dena-

turation of the enzymes occurring due to the rapid breakout of water molecules during depressurization [21]. The restrictor temperature was 65°C and the collection temperature 10°C. Aliquots of 1 ml were transferred to small vials for analysis.

*Extraction of food samples.* The supercritical conditions used for cleaning of enzyme preparations as well as for extraction of samples were as described above for the experiments with standard solutions. The supercritical solvent used was carbon dioxide containing 3 vol% of ethanol and 0.03 vol% of water, with the extraction performed at 80°C and a density of 0.8 g/ml. 1.0 g of immobilized enzyme was mixed with 0.5 g of hydromatrix and loaded into the extraction cell. Some glass wool was added on top in order to separate the enzyme preparation from the sample. After cleaning the enzyme preparation, the water activity was set to 0.43 as described above. 0.5 g of milk powder was mixed with 1 g of hydromatrix ($a_w$ = 0.43) and added on top of the glass wool and the enzyme preparation in the extraction cell. 2 ml of ethanol (containing 1% of water and 0.1% BHT) was added on top in order to achieve quantitative extraction of the vitamins [3], and the cell was filled up with hydromatrix ($a_w$ = 0.43). Infant formula was prepared similarly, except that 1 ml of 90°C water was added before mixing with hydromatrix, and only 1 ml of ethanol was added as entrainer. The direction of the flow was from the sample towards the enzyme bed. The collection solvent, which was the same as described above for the standard solutions, was evaporated under nitrogen in a water bath set to 40°C to a volume of approximately 1.5 ml, which was transferred to a smaller vial and taken to analysis.

## 3. Results and discussion

### 3.1. Chromatography

The HPLC conditions applied in this work allowed determination of retinol, retinyl palmitate, vitamin $D_2$, α-tocopheryl acetate, α-, β/γ- and δ-tocopherol in a single run within 60 minutes including re-equilibration of the system (see Materials and methods). The chromatogram in Fig. 2 shows the *Candida rugosa* lipase-catalyzed hydrolysis of retinyl palmitate and α-tocopheryl acetate in DIPE after 6 hours of reaction. The analysis of both substrates (the vitamin esters) and products (the vitamin alcohols) enables an accurate determination of the vitamin recovery and evaluation of the completeness of the enzyme-catalyzed reaction. All experiments performed in this work gave a total recovery of 100% when calculating the sum of recovered vitamin alcohol and its corresponding ester.

### 3.2. Screening of enzymes

Six different lipases from the major lipase families were investigated: the mammalian lipase PPL, the fungal lipases

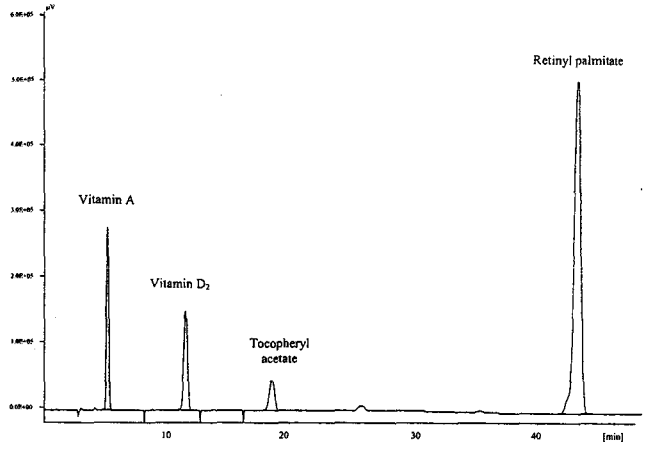


Fig. 2. HPLC chromatogram after 6 hours of CRL-catalyzed hydrolysis of retinyl palmitate and α-tocopheryl acetate in water-saturated di-isopropyl ether using variable UV detection at 325, 265, 284 and 325 nm (see Materials and methods).

CRL, CALB, RML and ROL and the bacterial lipase PCL. Also the esterase PLE was investigated. The catalyzed reactions were hydrolysis and alcoholysis of liposoluble esters with water and ethanol as nucleophiles, respectively. The vitamin esters produce active vitamin alcohols following any of these two reaction schemes, hence neither of them was regarded as an unwanted side-reaction. The water concentration of the reaction system was primarily optimized to assure fast enzyme reactions. Since lipases have their maximal activity at different water activities, solvent systems of different water content were investigated in order to find the most suitable enzyme. Moreover, since it is difficult to maintain constant water activity during a continuous supercritical extraction/reaction procedure, two extreme levels of water activity were investigated in organic solvents, 0.11 and 0.97, in order to cover the entire range. The results are shown in Fig. 3.

In Fig. 3a, DIPE containing approximately 1.2% (w:w) of water and immobilized enzymes of water activity 0.97 was applied, which should exclusively give hydrolysis. Evidently, all the examined enzymes except PPL and PLE catalyzed the hydrolysis of retinyl palmitate to retinol (vitamin A), while none of the enzymes showed any activity with α-tocopheryl acetate. For three of the enzymes, RML, PCL, and CALB, the reactions were fast giving recoveries above 90% within 6 hours.

Nevertheless, a second solvent was investigated, hexane/ethanol (87.5/12.5) of water activity 0.11, since this reaction media is more similar to supercritical carbon dioxide. Considering the low water activity of both solvent and enzyme (both were set to 0.11), the dominant reaction should here be alcoholysis with ethanol as nucleophile.

In Fig. 3b, with hexane/ethanol as solvent, the reaction is slower than that observed in DIPE, but the same three enzymes (RML, PCL and CALB) gave the highest reaction rates and recoveries of vitamin A. Moreover, the same two enzymes (PPL and PLE) plus CRL, gave no activity with

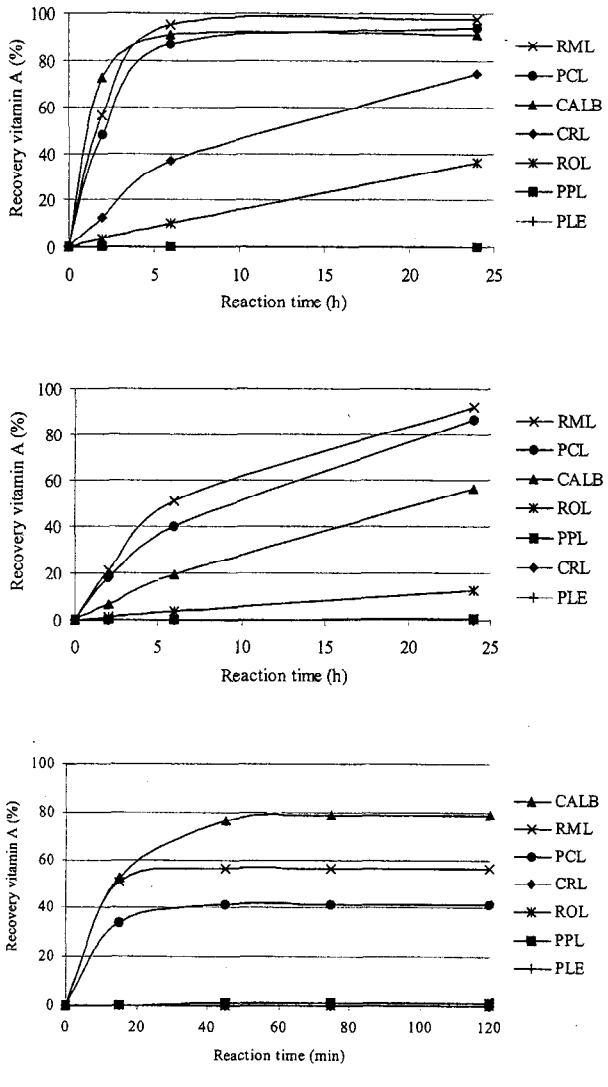


Fig. 3. Recoveries of vitamin A obtained by enzymatic hydrolysis and/or alcoholysis of retinyl palmitate using six different lipases (RML, PCL, CALB, CRL, ROL and PPL) and one esterase (PLE) in a. water-saturated DIPE, b. hexane/ethanol, 87.5/12.5 ($a_w$ = 0.11) and c. supercritical carbon dioxide containing 3 vol% of ethanol and 0.15 vol% of water, at 60°C and 260 bar. The water activities of the immobilized enzymes were set to 0.97, 0.11 and 0.43 in system a., b. and c., respectively. Each point in Fig. a. and b. represents the vitamin A concentration, recalculated to a recovery value, in the system after a certain reaction time. In Fig. c. on the other hand, each point gives the sum of the amount of vitamin A found in the different collection vials. Three replicate measurements were made.

retinyl palmitate. None of the enzymes catalyzed the reaction with α-tocopheryl acetate. One explanation to the lack of activity with this vitamin ester could be that the substrate is sterically hindered to enter the active sites of the investigated enzymes, as was pointed out in a recent paper [12]. Another explanation could be that the reaction is highly specific, which has been discussed by others [22].

In Fig. 3c the reaction medium was supercritical carbon dioxide. The water activity of the immobilized enzymes was set to 0.43, and the carbon dioxide contained 3 vol% of ethanol and 0.15 vol% of water. The reason for using a water activity of 0.43 instead of 0.97, even though experiments in organic solvents showed that a higher water activity gives faster reaction kinetic, was that under supercritical conditions too much of water on the support material may hinder the substrates (here retinyl palmitate and α-tocopheryl acetate) from reaching the enzymes [23]. However, this water activity will nonetheless change during the extraction with carbon dioxide, and will depend on several parameters such as ethanol concentration, temperature and pressure. Therefore, at least the change in water activity during the extraction was kept constant in the different experiments.

It is difficult to compare the reactions obtained in supercritical carbon dioxide (Fig. 3c) with those obtained in DIPE or hexane/ethanol (Fig. 3a–b), since in the supercritical system a larger amount of enzyme was used, which was applied as a packed-bed reactor. In addition, the reaction temperature was 60°C in the supercritical system, while it was only 20°C in the organic solvents. However, it seems reasonable that the mass transfer kinetics would be faster in a packed-bed system than in a batch system with stirring. Moreover, the higher diffusion coefficients in supercritical carbon dioxide compared to organic solvents also leads to increased internal mass transfer rates [24], which should improve the reaction rate as well as the extraction rate. In spite of this, the recoveries for SFE of vitamin A were lower than when using organic solvents. The main reason for this is most probably the much shorter contact time between the analyte and the enzyme in a dynamic SFE extraction system. Here the vitamin esters released from the sample are moving through the enzyme bed. That the reaction was the limiting factor could be ascertained by quantifying the retinyl ester, which had passed the extraction cell. Under the experimental conditions the enzyme kinetic was CALB>RML>PCL.

Provided that the recoveries could be increased and approach 100%, there are advantages with SFE compared to the two other procedures discussed. These include faster automated analysis, reduced use of organic solvents and less sample preparation. In enzymatic hydrolysis of food samples in liquid solvents additional solvent extraction is needed before determining the vitamins [10,25].

Hence, SFE was used as the preferred approach, and the experiments below describe our attempts to improve the recoveries by varying water content and temperature, parameters which have large influence on enzyme kinetics.

### 3.3. Optimization of supercritical conditions

Since the same three enzymes CALB, RML and PCL gave the highest recoveries of vitamin A in all solvents tried, they were chosen for further investigation at supercritical conditions.

#### 3.3.1. Cleaning

The first discovery that was made using the supercritical system was that the enzyme preparations contained liposol-

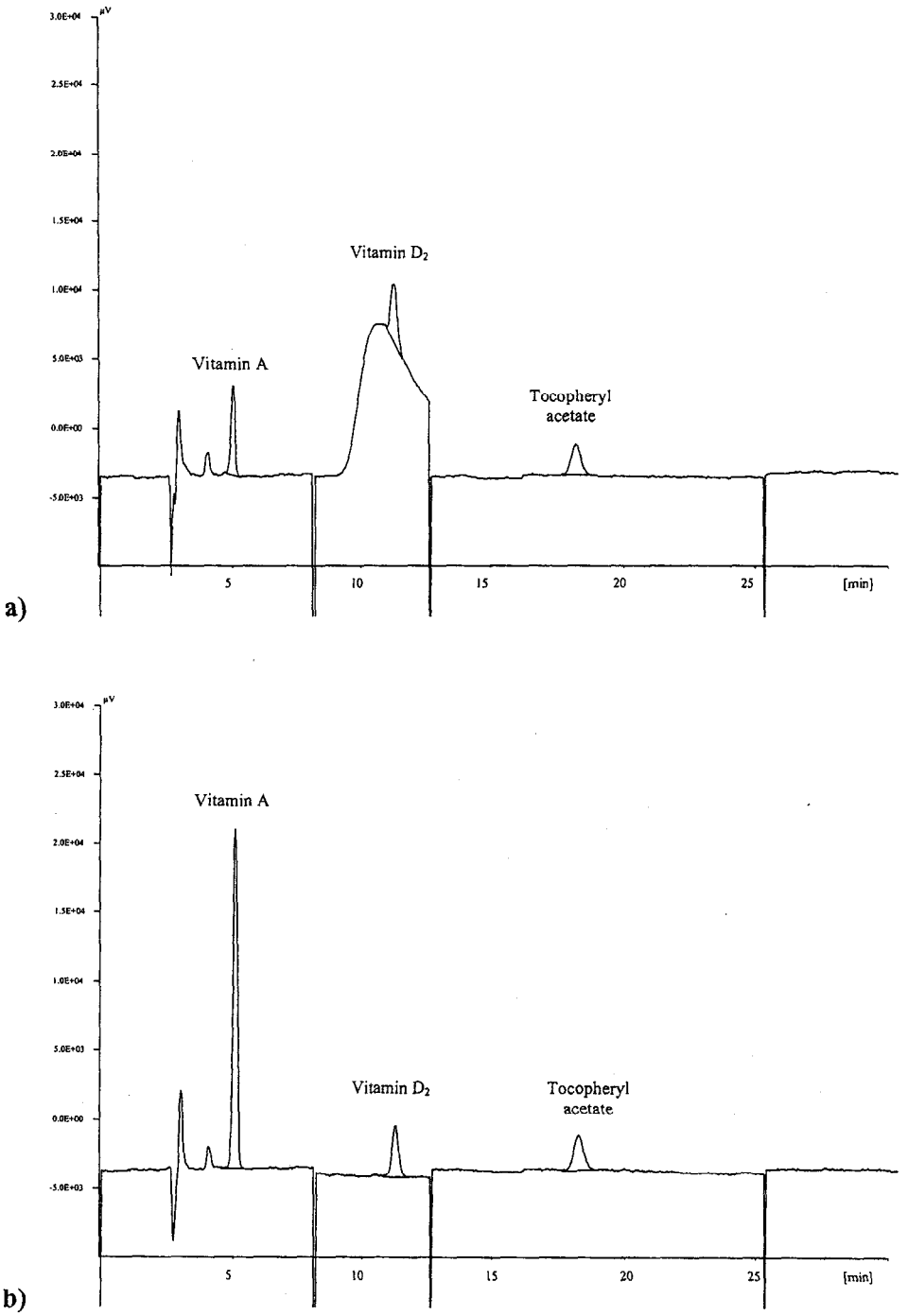


Fig. 4. HPLC chromatograms showing a. contaminants from unclean immobilized CALB appearing after 15 minutes of extraction using supercritical carbon dioxide containing 3 vol% of ethanol and 0.15 vol% of water, at 60°C, 260 bar, 0.8 g/ml and 0.5 ml/min, and b. the same enzyme preparation used after 30 minutes of cleaning with supercritical carbon dioxide applying the same conditions as in a.

uble components, which disturbed the chromatographic analysis. These compounds were not observed, when the reaction was performed in organic solvents, most likely due to that only 20 mg of enzyme was used instead of the 0.5 g applied in the supercritical setup. It is also possible that supercritical carbon dioxide modified with ethanol more efficiently extracts these compounds from the support material, compared to hexane/ethanol or DIPE. A chromatogram showing the first collection vial after 15 minutes of extraction/reaction using unclean CALB is presented in Fig. 4a.

As shown in Fig. 4a, there is a large component eluting at the same time as the internal standard, vitamin $D_2$, which was detected at 265 nm. The component was suspected to be several coeluting lipids, since even stronger absorption was found at 210 nm, and a homologous series of more than 15

Table 1
Recoveries of vitamin A obtained by enzymatic reaction of retinyl palmitate using CALB, RML and PCL in supercritical carbon dioxide containing 3 vol% of ethanol and different concentrations of water (n = 3). The recovery values were calculated from the total amount of vitamin A found in the four separate collection vials. RSD values are shown within the parentheses.

| Water content (vol%) | Vitamin A recovery (%) | | |
|---|---|---|---|
| | CALB | RML | PCL |
| 0.03 | 87 (4) | 11 (22) | 79 (4) |
| 0.09 | 80 (19) | 62 (22) | 49 (1) |
| 0.15 | 79 (3) | 57 (8) | 41 (12) |

peaks was obtained by GC/FID analysis. The problem arose with enzymes immobilized in our own laboratory as well as with commercially purchased immobilized lipases, which indicated that the liposoluble components were adsorbed onto the support material during the immobilization step. It has been found by others that crude PPL contained significant amounts of free fatty acids, which could be removed using supercritical carbon dioxide. This increased the activity of the enzyme 8.6 times compared to unclean crude PPL [26]. In this work, a 30 minutes long cleaning procedure with supercritical carbon dioxide of the same conditions as applied during the enzymatic reaction was required before sample was loaded into the extraction cell. This procedure gave sufficiently clean enzymes as shown in Fig. 4b. No losses in activity were observed.

### 3.3.2. Water content

The water content of the support material strongly affects the activity of the enzyme, which in turn depends on the distribution of water between the supercritical fluid and the support material [27]. The water content of the support material under certain supercritical conditions may be determined by measuring water sorption isotherms using special SFE instrumentation [28]. Several authors have found that a water content of 8–10% (w:w) regardless of solvent type is optimal for esterification reactions using RML [23, 27] or PPL [28]. Since the resulting water activity depends on the type of support material and the optimal water activity usually differs between different enzymes and type of reaction catalyzed, this has to be optimized for each application. Hence, in order to enable comparison between CALB, RML and PCL, three different levels of water concentration of the supercritical fluid were examined: 0.03, 0.09 and 0.15 vol%. The choice of level was determined by observations that higher water concentrations could lead to reduced enzymatic activity or even denaturation of the enzyme [23]. The water activity of the immobilized enzymes was equilibrated to 0.43 before each experiment performed. The results are shown in Table 1.

The results in Table 1 show that especially RML and PCL are greatly affected by the water content of the solvent, but in a reverse manner. The RML-catalyzed reaction seems to improve with increasing water concentration, while PCL works best at the lowest water concentration. However, CALB gives high vitamin A recovery at all water concentrations examined, with a tendency of improvement towards lower concentrations. The results for RML agree with what was found by Dumont et al., i.e. that the activity of RML increased up to a water concentration of 0.25% (w:w, which corresponds to 0.20 vol% at 0.8 g/ml), and then it dropped [29]. The highest vitamin A recovery for PCL found at the lowest water concentration using SFE contradicts results obtained in organic media [30]. However, it has been found that under supercritical conditions (520 bar, 75°C), PCL lost 50% of its activity, when of 1 mole% of water was added to the carbon dioxide [31]. Hence, it is most likely that the higher stability of PCL at low water activity is a good explanation to the SFE results.

Table 2
Recoveries of vitamin A obtained by enzymatic reaction of retinyl palmitate using CALB and RML in supercritical carbon dioxide containing 3 vol% of ethanol and 0.15 vol% of water at 40, 60 and 80°C, respectively (n = 3). The water activities of the immobilized enzymes were set to 0.43 and the density was maintained at 0.8 g/ml by using different pressures for the temperatures investigated. The recovery values were calculated from the total amount of vitamin A found in the four separate collection vials. RSD values are shown within the parentheses.

| Temperature (°C) | Vitamin A recovery (%) | |
|---|---|---|
| | CALB | RML |
| 40 | 80 (5) | 20 (7) |
| 60 | 83 (5) | 57 (8) |
| 80 | 89 (4) | 3 (21) |

### 3.3.3. Temperature

Higher temperature generally gives faster reaction rates, but may also accelerate unfolding processes of the enzyme, especially if the enzyme contains large amounts of water [32]. The temperature effects and stability of different enzymes in supercritical media vary considerably, and has been investigated by several authors [31–33]. In this work, CALB and RML were utilized at 40, 60 and 80°C, and the results from these experiments are shown in Table 2.

The results in Table 2 clearly demonstrate the high temperature stability of CALB in supercritical carbon dioxide containing 3 vol% of ethanol and 0.15 vol% of water. It has already been shown by Overmeyer et al. that Novozyme 435 (CALB) is stable in supercritical carbon dioxide at temperatures above 100°C, with the highest activities at 90°C [32]. RML on the other hand, gains activity when increasing from 40 to 60°C, but is thermally denaturated at 80°C. These results agree with results obtained by Habulin et al. [34], where RML catalyzed esterification performed in organic media gave increasing yields from 20°C to 50°C, with a drop in activity at higher temperatures. The increasing vitamin A recoveries at higher temperatures for CALB could

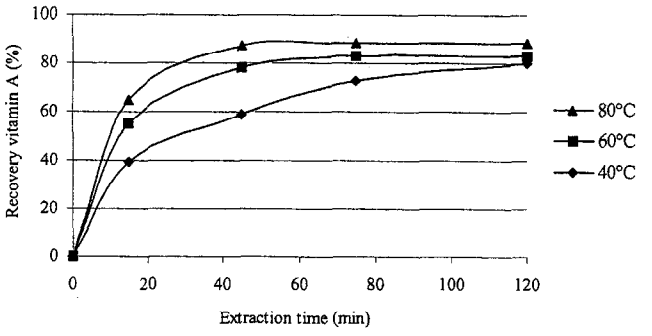


Fig. 5. Recoveries of vitamin A obtained by CALB-catalyzed reaction in supercritical carbon dioxide containing 3 vol% of ethanol and 0.15 vol% of water at 40, 60 and 80°C (n = 3). The density was maintained at 0.8 g/ml at all temperatures investigated.

be explained by both faster mass transfer of the analyte to the active site of the enzyme and by higher reaction rates, as illustrated in Fig. 5. The transformation rate of retinyl palmitate to retinol obviously increases significantly with increasing temperature in the interval 40–80°C.

After taking the obtained results into consideration, immobilized CALB was chosen as catalyzing enzyme, together with an extraction/reaction temperature of 80°C and a water concentration of 0.03 vol%. At this relatively high extraction/reaction temperature, it is advantageous to keep the water concentration as low as possible in order to minimize the risk of thermal denaturation of the enzyme [23]. An experiment using these parameters was performed for confirmation of the optimum, giving 89% recovery of vitamin A (n = 3, RSD = 1%), with an extraction time of 5 minutes static and 15, 30, 30 and 45 minutes dynamic extraction. The pressure was 366 bar, giving a density of 0.8 g/ml. 3 vol% of ethanol (containing 1 vol% water, thereby providing the supercritical fluid with 0.03 vol% water) was used as modifier during the first 110 minutes of dynamic extraction. These parameters were used for further experiments with real food samples.

### 3.4. Extraction of food samples

It was found that 1 g of immobilized CALB was needed in order to quantitatively transform retinyl palmitate to retinol as well as to hydrolyze most of the coextracted triacylglycerols from the milk powder. This is most likely due to the relatively high fat content of both milk powder and infant formula (approximately 24%, w:w), which results in a competition between the glycerol esters and the retinyl esters about the active sites of the enzyme.

#### 3.4.1. Cleaning and reusing

The possibility of reusing the enzyme was investigated with experiments performed both with standard solution of retinyl palmitate (1 ml of 99% ethanol containing 10 μg of retinyl palmitate) and with milk powder (0.5 g containing ca 1 μg of retinyl palmitate). CALB was cleaned in-between extractions with supercritical carbon dioxide as described in Materials and methods, and re-equilibrated to a water activity of 0.43. The enzyme preparation was not removed from the extraction cell for cleaning or re-equilibration, but the lid was taken off during the latter. New sample (or standard solution) was taken each time, and the old one was carefully removed after the extraction. The results are shown in Table 3.

Table 3
Recoveries of vitamin A obtained by cleaning and reusing the same immobilized CALB in supercritical carbon dioxide, applied to standard solutions of retinyl palmitate as well as milk powder. The supercritical conditions used for extraction/reaction were for the milk powder: supercritical carbon dioxide containing 3 vol% of ethanol and 0.03 vol% of water, at 80°C and 366 bar, and for the standard solutions as described in Materials and methods. The cleaning procedure used the same supercritical conditions as applied for extraction/reaction, except that the total extraction time was 30 minutes and the flow rate 2 ml/min. The recovery values were calculated from the total amount of vitamin A found in the four separate collection vials. RSD values are shown within the parentheses (n = 3).

| Order of using | Vitamin A recovery (%) | |
|---|---|---|
| | Standard solution | Milk powder |
| 1 | 81 (14) | 110 (6) |
| 2 | 79 (3) | 107 (10) |
| 3 | 77 (5) | 110 (10) |
| 4 | 76 (5) | 101 (15) |

The results in Table 3 indicate that immobilized CALB can be reused at least three times without significant differences in catalytic capacity. The enzyme preparation can most likely be reused even more times. It was found by Snyder et al. [35] that this enzyme preparation (Novozyme 435 from Novo Nordisk) could be reused at least 25 times for transesterification of lipids, while maintaining quantitative yields, in supercritical carbon dioxide containing 0.5 vol% methanol, at 172 bar and 50°C. An explanation to the higher vitamin A recoveries obtained in milk powder compared to those obtained using standard solutions in Table 3, could be that the concentration of retinyl palmitate was ca 10 times lower in the applied milk powder than in the standard solution. Moreover, the release of retinyl palmitate is slower from the milk powder compared to a standard added on Hydromatrix in the cell. This means that even with the same total amount of retinyl palmitate in the milk powder as in the added standard solution, the concentration of the ester in the supercritical fluid passing the enzyme bed will be lower. This will of course also facilitate the enzymatic transformation process.

#### 3.4.2. Extraction time

When extracting real food samples there will be a slow release of the vitamins and their esters from the sample, which might lead to a need of increased extraction times. However, with extraction times used in this paper this was not supposed to become a large problem, taking into ac-

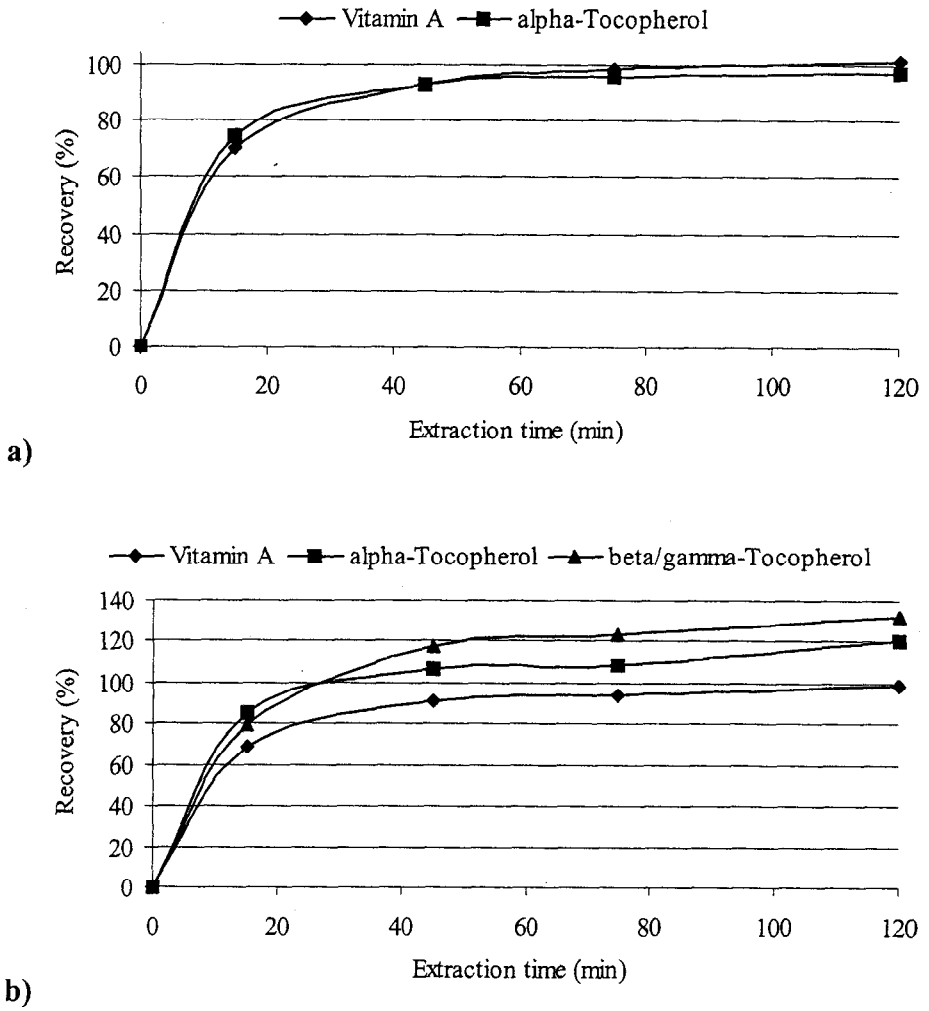


Fig. 6. Simultaneous CALB-catalyzed reaction and supercritical fluid extraction of vitamins A and E (tocopherols) from a. milk powder and b. infant formula, using 1 g of immobilized CALB and supercritical carbon dioxide containing 3 vol% of ethanol and 0.03 vol% of water, at 80°C, 366 bar and 0.8 g/ml (n = 3). The flow rate was changed from 0.5 ml/min to 1.0 ml/min after 75 minutes of extraction. 100% carbon dioxide was used during the last 10 minutes.

count previous experiences of vitamin extractions from food samples without using enzymes for hydrolysis [3,4]. Here 60 minutes turned out to be sufficient with 5% of ethanol as modifier. Nevertheless, this was checked for milk powder and infant formula, since the modifier concentration was lower. In Fig. 6 the recoveries for the different vitamins have been plotted versus extraction time.

When comparing the results for milk powder in Fig. 6a with the curve for 80°C in Fig. 5, it is clear that the extraction profiles are almost identical. It can therefore be concluded that the reaction is fast enough to assure quantitative recoveries of vitamin A also in the presence of large amounts of glycerol esters, and 60 minutes of dynamic extraction time would be sufficient for the considered vitamins. The results for infant formula given in Fig. 6b show that 120 minutes of extraction/reaction is needed for quantitative determination of vitamin A and that the recoveries of the tocopherols increase at the end of the extraction. This deviating increment is still important even when taking into consideration the higher flow rate at the end of the extraction. This thus most likely depends on that 100% carbon dioxide is used as extraction medium the last 10 minutes of the extraction. It seems advantageous to use a less polar solvent in order to achieve a total extraction of the tocopherols, which are tightly associated with the highly lipid containing matrix. This has earlier also been found for extraction of β-carotene from the same sample [17].

In order to determine the total vitamin E content in food samples, possible amounts of α-tocopheryl acetate have to be quantified together with the tocopherols, since none of the investigated enzymes, including the preferred CALB, catalyzed the hydrolysis of this vitamin ester. However, the determination of α-tocopheryl acetate is not demanding, since its retention time in a reverse phase system typically is around two minutes longer than that of α-tocopherol (in this paper 18 and 16 minutes respectively), and the quantification is easily achieved using UV detection at 284 nm.

The results for milk powder and infant formula using CALB are given in Table 4, together with results obtained

Table 4
Determination of fat-soluble vitamins in milk powder and infant formula using immobilized CALB in supercritical carbon dioxide, using the same conditions as described for milk powder in Table 3 (n = 3). The reference values were obtained within an Intercomparison study using the same samples, where ten laboratories used SFE with subsequent saponification and two laboratories used conventional saponification/solvent extraction [17].

| | Milk powder | | Infant formula | | | |
|---|---|---|---|---|---|---|
| | Vitamin A | α-Tocopherol | Vitamin A | α-Tocopherol | β/γ-Tocopherol | δ-Tocopherol |
| Average (mg/100 g) | 0.142 | 0.303 | 0.404 | 7.949* | 4.454 | 1.638 |
| Recovery (%) | 112 | 101 | 96 | 119 | 130 | — |
| RSD | 12 | 10 | 8 | 5 | 6 | 7 |
| n | 15 | 12 | 6 | 6 | 6 | 6 |
| Reference value (mg/100 g) | 0.127 | 0.299 | 0.419 | 6.694 | 3.421 | — |

* This value is a sum of α-tocopherol (2.556 mg/100 g) and α-tocopheryl acetate recalculated to α-tocopherol (5.393 mg/100 g).

in the Intercomparison study using samples from the same batch [17].

The vitamin concentrations found using the method described in this work are similar or even higher than those obtained using SFE with saponification or conventional hydrolysis/extraction techniques. The higher values reported using the enzyme-SFE technique may reflect a more gentle treatment, and for tocopherols in infant formula also the use of 100% carbon dioxide the last 10 minutes of the extraction as discussed above. The SFE method used to obtain the reference values, on the other hand, included an unpolar extraction step (100% $CO_2$) at the beginning of the extraction, then followed by a more polar step ($CO_2$/methanol (95:5)). However, it should be more advantageous though to initiate the extraction with the more polar solvent, in order to enable disruption of the sample matrix, and thereafter use an extraction solvent that is suitable regarding to the analytes solubility in the solvent.

The listed RSD values are good taking into consideration that these variations include between-day experiments for both food samples, and reusing of the enzyme for milk powder. Furthermore, the vitamin concentrations in the samples are low (ca 1 to 50 ppm).

### 3.4.2. Concluding remarks

The final method used for SFE based determination of vitamins A and E in milk powder and infant formula turned out to be similar to the one recently presented [12]. None of the other considered enzymes in this paper proved to be better than the lipase from *Candida antarctica* type B. In this paper, the importance of optimizing and controlling the water concentration during the extraction/reaction procedure has been emphasized. This was one source of uncertainty with the previous method [12], which now has been markedly reduced. Moreover, we have now also shown that a higher temperature improves the lipase-catalyzed reaction of retinyl palmitate, and that a longer total extraction time with 100% carbon dioxide the last 10 minutes considerably improves the vitamin recoveries from infant formula.

### 3.5. Conclusions

The hydrolysis/alcoholysis of retinyl palmitate to retinol in di-isopropyl ether, hexane/ethanol and supercritical carbon dioxide was catalyzed by primarily three of the screened seven enzymes, lipases from *Rhizomucor miehei*, *Pseudomonas cepacia* and *Candida antarctica B*, of which the latter showed outstanding performance at supercritical conditions. The advantages of using supercritical carbon dioxide rather than organic solvents for analytical purposes are faster automated analysis with less sample preparation and lower total consumption of organic solvents. Using the optimized supercritical conditions, immobilized *Candida antarctica* type B gave quantitative conversion of retinyl palmitate to retinol, and was successfully applied in automated SFE for determination of vitamins A and E in milk powder and infant formula, without any need of further clean-up. Since α-tocopheryl acetate is not hydrolyzed by any of the enzymes, it must and can easily be determined separately and included in the vitamin E recovery. The analytical methodology developed is faster and much more beneficial to the oxidation prone vitamins than extraction methodologies based on alkaline saponification. It should be applicable for the determination of vitamins $D_2$/$D_3$, $K_1$ and β-carotene as well.

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